

# Accuracy of the functional, flow cytometer-based Emo-Test HIT Confirm® for the diagnosis of heparin-induced thrombocytopenia

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## SUMMARY

**Introduction:** Rapid functional assays have been proposed to overcome the limitations of washed platelet assays in the work-up of patients with suspected heparin-induced thrombocytopenia (HIT). Data on the diagnostic accuracy are, however, scarce and conflicting. We aimed to study the diagnostic accuracy of a rapid, flow cytometer-based assay and to explore sources of variability.

**Material and methods:** Frozen serum samples of 103 consecutive patients, evaluated for suspected HIT at our institution in 2017, and characterized with 4Ts score, IgG-PF4/heparin ELISA (GTI), HemosIL®Acustar (IgG), as well as heparin-induced platelet activation test (HIPA), were further tested using HIT Confirm, determining P-selectin release of donor platelets after incubation with patient's serum. The diagnosis of HIT was defined as a positive HIPA result.

**Results:** HIT was confirmed in 15 out of 103 patients corresponding to a prevalence of 14.6%. HIT Confirm was positive in 11 patients (10.7%), negative in 88 patients (85.4%), and inconclusive in 4 patients (3.9%). According to the intention-to-diagnose principle, the number of true positives was 9, the number of true negatives 83, the number of false negatives was 6, the number of false positives 5. This corresponds to a sensitivity of 60.0%, and a specificity of 94.3%. Modifications of the test did not improve sensitivity.

**Conclusions:** The rapid, flow cytometer-based assay HIT Confirm is able to verify HIT in positive patient samples but cannot rule-out HIT in clinical practice. Other rapid functional assays shall be studied in appropriately designed diagnostic accuracy studies.

## 1. Introduction

Because of the complex mechanism of heparin-induced thrombocytopenia (HIT), the diagnostic work-up is challenging. HIT is an immunological disorder caused by the development of antibodies that recognize platelets factor 4 (PF4) complexed with polyanions such as heparin (anti-PF4/ H abs). These immune complexes bind to FcγIIA-receptors, trigger activation of platelets, support derivation of platelet microparticles, and, subsequently, accelerate thrombin generation [1]. Well-known clinical consequences are thrombocytopenia and massive thromboembolism. Immunoassays such as enzyme-linked immunosorbent assay (ELISA) or chemiluminescence immunoassay (CLIA) [2] can quantify anti-PF4/H antibodies with high sensitivity [3]. However, immunoassays cannot distinguish antibodies that activate platelets and others that do not [4–6]. Besides, the diagnostic accuracy depends on

the type of the antibody, diagnostic thresholds, and manufacturers [7]. Thus, functional assays are needed to demonstrate anti-PF4/H antibodies' capability to activate platelets [1].

Functional assays demonstrate the activation of donor platelets in the presence of anti-PF4/H antibodies and heparin [4,8–11]. Donors' platelets are stimulated either in whole blood (WB), platelet-rich plasma (PRP), or after washed platelets (WP) [10]. Washed platelet assays, the serotonin release assay (SRA) [12], and the heparin-induced activation test (HIPA) [9], are regarded as reference (gold) standard [13] to diagnose HIT. They appear sensitive and specific because the test does not utilize donors' PRP, affecting the reaction [3]. However, these tests are expensive, time-consuming, and required a particular laboratory infrastructure [3]. Rapid functional tests overcoming these shortcomings have been developed, but the diagnostic accuracy is still an open issue. Examples include heparin-induced platelet aggregometry (PAT) [14],

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and multiple electrode platelets aggregometry (Multiplate®) [15]. Even though some agreement with washed platelet assays were observed, the accuracy was clearly inferior in some cases [14]; others require further standardization and appropriate diagnostic accuracy studies [3]. Recently, the new cytometer-based test HIT Confirm® assessing the P-selectin (CD62P) release of donor platelets surface after incubation with patient's serum was developed. Few evaluation studies are available, but the results are conflicting [16–18].

With the present study, we aimed to comprehensively assess the diagnostic accuracy of the rapid, flow cytometer-based functional assay HIT Confirm and to explore sources of variability.

## 2. Material and methods

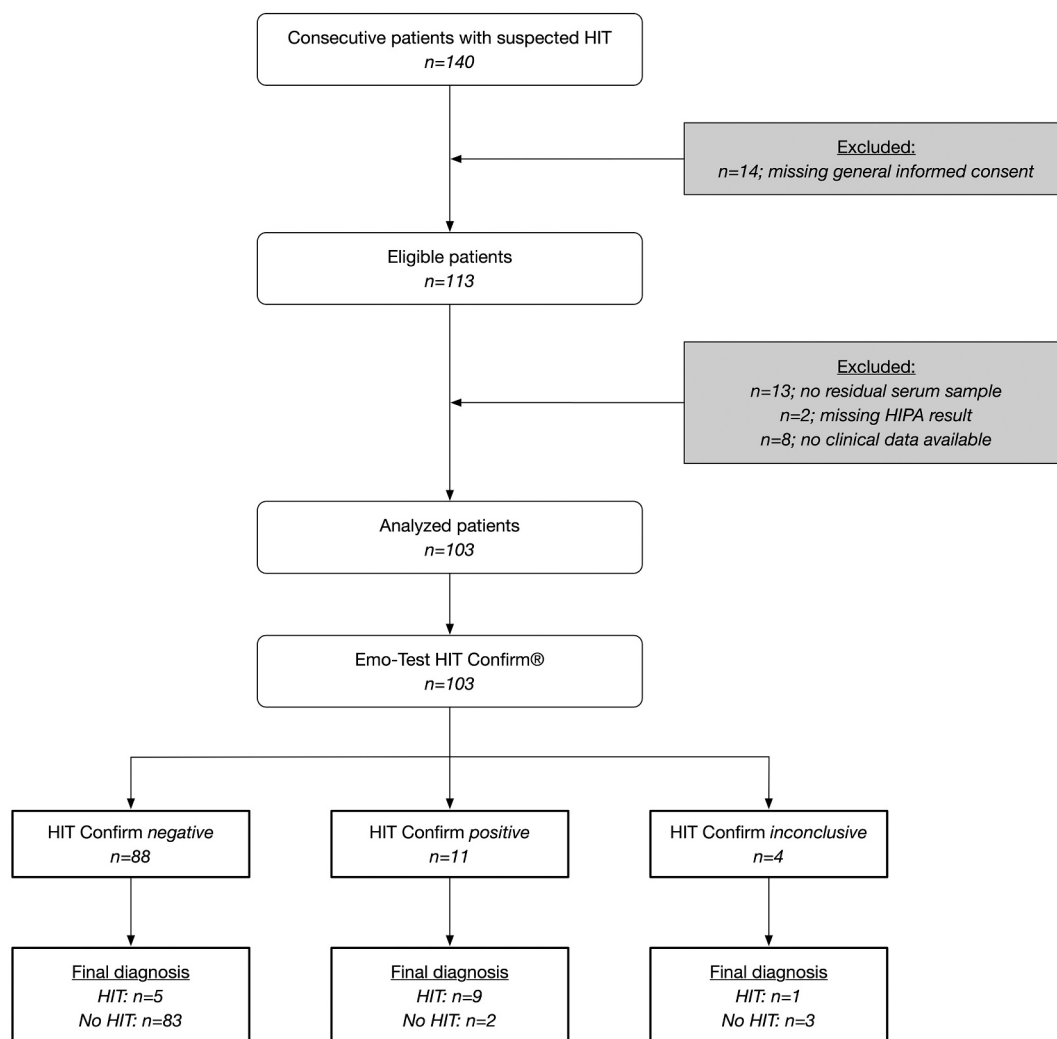
### 2.1. Design, setting and population

The present cross-sectional analysis was conducted in-line with a large prospective cohort study (TORADI-HIT). One-hundred and forty consecutive patients referred with suspected HIT to a specialized laboratory of a University hospital in 2017 were screened. The flow of the patients is shown in Fig. 1. HIPA was conducted if not done in clinical practice; ELISA and AcuStar were additionally completed. Inclusion criteria were (1) referred with suspected HIT, (2) 4Ts score rated by the

consultancy team (at least clinical characteristics available), (3) immunoassay test results available, (4) residual serum samples available, (5) age above 18 years, and (6) signed general informed consent. Inselspital University Hospital is a large tertiary hospital in Switzerland covering a catchment area of more than 1.5 million inhabitants. The appropriate ethical committee approved the protocol of the study. The study was conducted in accordance with the declaration of Helsinki.

### 2.2. Collection, storage and handling of samples

For practicability reasons, plasma samples were obtained to determine AcuStar HIT IgG and IgG ELISA, and serum samples to conduct HIT Confirm and HIPA. A standardized protocol was implemented to ensure adequate preanalytic conditions [14,19]. Samples were collected into 3.2% sodium citrate (4.3 mL S-Monovette, Sarstedt Nümbrecht, Germany) and serum (4.7 mL S-Monovette, Sarstedt Nümbrecht, Germany) containers. Platelet-poor plasma was obtained by double centrifugation at 1500 ×g for 10 min at room temperature; serum was centrifuged by 3137 g for 7 min [20]. Samples were immediately snap-frozen at −20 °C and stored at −80 °C. AcuStar HIT IgG was conducted before freezing; ELISA and HIPA were conducted in batches every other day.



**Fig. 1.** Design of the cross-sectional diagnostic accuracy study. Consecutive patients referred with suspected HIT were included, and Emo-Test HIT Confirm® conducted. Heparin-induced platelet aggregation test was used as the reference standard. HIPA, heparin-induced platelets assay; HIT, heparin-induced thrombocytopenia.

### 2.3. Collection of clinical data and determination of immunoassays

Clinical characteristics were collected by the hematology consultation service, which is activated in every case a HIT antibody test is requested. In-line with this consultation, a 4Ts score is rated. For this analysis, these data were retrieved from the electronic patient documentation. HemosIL AcuStar HIT-IgG (Instrumentation Laboratory, Bedford, MA, USA) was conducted using citrated plasma samples 24/7 as the time of suspected HIT on a BIO-FLASH1 analyzer (Inova Diagnostics, San Diego, California, USA). The determination was done according to the manufacturer's instructions. An IgG-specific ELISA was conducted using plasma samples in batches every other day (Immunocor Lifecode PF4 IgG) according to the manufacturer's instructions.

### 2.4. Preparation of platelet-rich-plasma, (PRP) donor and patients' samples

Whole blood of five unselected healthy donors was collected into acid-citrate-dextrose solution (ACD) containers to prevent platelet aggregation and bind residual calcium. The material was prepared within 3 h after collection. PRP was prepared by centrifugation at 120 ×g for 20 min without brake, at room temperature [9] after resting for 30 min. Patient samples were prepared by thawing for 5 min at 37 °C and heat-inactivated by 56 °C for 45 min. Then, samples were centrifuged by 10,000 ×g for 5 min [14].

### 2.5. Determination of Emo-Test HIT Confirm® done on PRP

The Emo-test HIT Confirm® assay (Emosis SAS, Illkirch-Grattenstaden, France) was performed according to the manufacturer's instructions [10,16]. First, an initial mix was generated using 50 µL donor PRP as well as 115 µL dilution buffer and labeled with 5 µL antibodies (CD41-PE and CD62P-FITC). Then, we incubated 35 µL of the mix, 10 µL of patient's serum, and positive control material (activated with at thrombin receptor activating-peptide [TRAP]) and negative control material (dilution buffer) in the presence of heparin (0.3 IU/mL or 100 IU/mL respectively) in the dark. Following 30 min of incubation at room temperature, stimulation was stopped using 450 µL of dilution buffer. Samples were immediately analyzed by BD FACSCANTO™ II Flow Cytometer (BD Biosciences, San José, CA 95131 USA). CD41/CD62 surface expression was measured. The percentage of CD62P+ platelets was measured for 3 IU and 100 IU heparin, respectively, by analyzing 10'000 events of CD41+ platelets. The cutoff was defined at the intersection of the negative and positive control of the FITCCD62P histogram. The percentage of CD62P positive events was defined as the platelets activation index (HEPLA):  $[(R\% \text{ UFH } 0.3 - R\% \text{ UFH } 100)] / (R\% \text{ positive control} - R\% \text{ negative control}) \times 100$ . The cutoffs defined by the manufacturer were used ( $\geq 13\%$ , positive result;  $\geq 9.6\%$  inconclusive results).

### 2.6. Test modifications

We hypothesized that various test modifications might improve the diagnostic performance of HIT Confirm: (1) use of washed platelets rather than PRP, (2) using platelets of a second donor, and (3) filtrating the patient's serum. The number of experiments was restricted because of the limited amount of residual patients' material.

Nine patient samples with false-negative or inconclusive results in the HIT Confirm assay were selected and re-tested with washed platelets using various donors. The process of washing platelets is described below [4,8,14].

Eight patient samples with false-negative or inconclusive results in the HIT Confirm assay were re-tested with a second, independent platelet donor.

Ten patient samples with false-negative or inconclusive results in the HIT Confirm were re-tested after filtration of patients' serum. The

filtration was done using a spin -X Centrifuge Tube filter cellulose acetate of 0.22 µm (Costar®, Salt Lake City, USA).

### 2.7. Determination of HIPA test

HIPA test was performed as described previously [4,9,14,16]. Each serum sample was tested with four different washed platelets donor in the absence (buffer) and in the presence of different heparin concentrations (0.2 IU, 100 IU/mL) in a 96-microwells plate. PRP was prepared as mentioned above, and the platelets were washed as follows: the first pellet was re-suspended using calcium- and magnesium-free Tyrode's buffer at pH 6.3 (adding glucose and apyrase); the second pellet was re-suspended using calcium- magnesium-containing Tyrode's buffer at pH 7.2, and the suspension was incubated at 37 °C for 45 min. After thawing (5 min at 37 °C) and heating to inactivate residual thrombin (45 min at 56 °C), the patient's samples were transferred to the microplate well, adding platelet suspension and buffer or heparin, respectively. The microplate was incubated on a magnetic stirrer plate with two steel balls per well approximately by 600 rpm. The plate was examined every 5 min against the light. A patient was interpreted as positive if an aggregation of the platelets at least in two donors occurs within 30 min in the presence of 0.2 IU/mL of heparin, but not in the presence of 100 IU/mL heparin. Interpreting time is 45 min, and each test plate included positive and negative control.

### 2.8. Statistical analysis

Descriptive statistics were used to characterize the study population and describe the distribution of test results (median and IQR/ ranges or frequencies as appropriate). Diagnostic accuracy of the Emo-test HIT Confirm® assay was determined by calculating the sensitivity, the specificity, and the likelihood ratio in relation to the presence of HIT (defined as a positive HIPA test). Following the intention-to-diagnose principle, we created 3 × 2 tables and counted inconclusive results as negative in HIT positive patients and positive in HIT negative patients [21]. A logistic regression analysis fitting a maximum-likelihood dichotomous model was performed to explore the extent to which HIT Confirm contributes to the diagnostic process. HIPA result was used as the dependent variable, ELISA results, and HIT Confirm as independent variables. Analyses were performed using the Stata 14.1 statistic software package (StataCorp. 2014. Stata Statistical Software: Release 14. College Station, TX: StataCorp LP).

## 3. Results

### 3.1. Patient characteristics

Out of 140 patients screened, 37 were excluded because of missing data, refused informed consent, or insufficient sample material (Fig. 1). The median age was 69 years (interquartile range [IQR] 60 to 76), 39% of the patients were female. The median 4 T's score was 5 points in patients with HIT (IQR 3.5, 6), and 3 points in patients without HIT (IQR 2, 4). The median IgG-specific ELISA was 3.0 in patients with HIT (OD; IQR 2.1, 3.3) and 0.2 in patients without HIT (0.15, 0.4). HIT was confirmed by a positive HIPA test in 15 patients (14.6%), a number that was higher than expected. Indeterminate results with the HIPA test were not observed. Detailed patient characteristics are given in Table 1.

### 3.2. Diagnostic accuracy of HIT confirm

The distribution of HIT Confirm test results in HIT positive and HIT negative patients is illustrated in Fig. 2 (panel A). Among the 15 patients with confirmed HIT, nine samples tested positive with HIT Confirm (% HEPLA  $> 13$ ), resulting in a true positive rate (sensitivity) of 60% (95% confidence interval [CI] 32.3, 83.7). HIT Confirm tested negative in five patients (%HEPLA  $< 9.6$ ), and the result was inconclusive in one patient

**Table 1**

Baseline characteristics of the study cohort ( $n = 103$ ). Clinical characteristics were collected by the hematology consultancy service, and a 4Ts score was rated. HIT was defined as a positive heparin-induced platelet activation test (HIPA). Abbreviations: HIT, heparin-induced thrombocytopenia; IQR, interquartile range; OD, optical density; ELISA, enzyme-linked immunosorbent assay.

	HIT positive $n = 15$ (14.6%)	HIT negative $n = 88$ (85.4%)	All patients $n = 103$ (100%)
Age in years, median (IQR)	73 (62, 77)	69 (59, 76)	69 (60, 76)
Sex, numbers (%)			
Male	60 (58.3)	64 (62.1)	63 (61.2)
Female	40 (38.7)	36 (37.9)	37 (38.8)
4T's score, median (IQR)	5 (3.5, 6.0)	3.0 (2.0, 4.0)	3.0 (2.0, 4.0)
IgG-specific GTI ELISA, median OD (IQR)	3.0 (2.1, 3.3)	0.2 (0.15, 0.4)	0.27 (0.15, 0.60)
HemosIL®Acustar (IgG), median U/mL (IQR)	15.3 (2.3, 38.8)	0.0 (0.0, 0.0)	0.0 (0.0, 0.0)

(%HEPLA 9.6 to 13.0). Among HIPA negative patients, two tested positive for HIT Confirm, and three were inconclusive. The specificity was thus 94.3% (95% CI 87.2, 98.1). The results are illustrated in Fig. 2 (panel B).

We further analyzed the diagnostic accuracy of HIT Confirm within different risk categories of the 4Ts score. In patients at low risk, the sensitivity was 0% (95% CI 0% to 71%) and the specificity 98% (89, 100). At intermediate risk, the sensitivity was 50% (7, 93), and the specificity was 97 (85, 100). At high risk, the sensitivity was 88% (47, 100), and the specificity was 100% (40, 100).

To contrast the accuracy of HIT confirm with ELISA's accuracy, we calculated the sensitivity of ELISA IgG in our dataset (100%, 95%CI 75, 100), and the specificity (77% 95%CI 66, 85). The numbers of true positives, false negatives, false positives, and true negatives were 13, 0, 20, and 66.

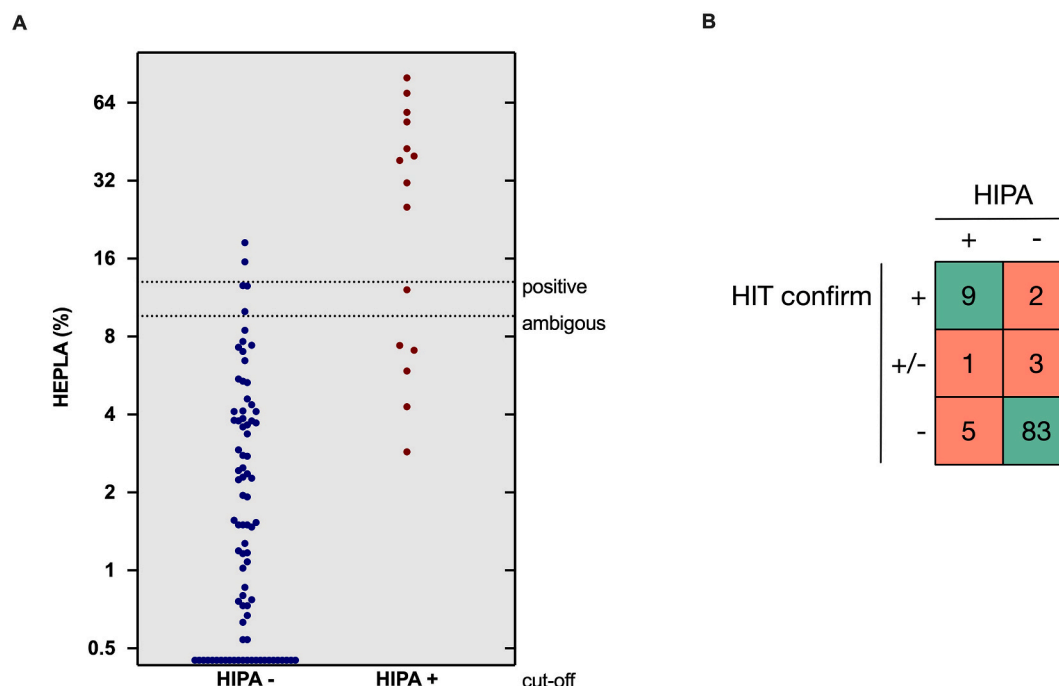
To explore how HIT confirm contributes to the diagnostic process, we conducted a logistic regression analysis. Whereas the odds ratio was 23.1 for ELISA (95% CI 3.3, 165.2), it was 1.2 for HIT Confirm (95%CI 0.9, 1.6;  $R^2 = 0.8$ ;  $p < 0.001$ ).

### 3.3. Test modifications

We re-tested several samples with inconclusive results (A) using washed platelets rather than PRP, (B) using platelets of a second donor, and (C) filtrating the patient's serum to explore the potential effects of test modifications on the diagnostic performance. No systematic improvement of the results was observed, and the diagnostic performance was similar.

## 4. Discussion

In a cross-sectional diagnostic accuracy study, we included 103 consecutive patients referred with suspected HIT to study the performance of a rapid, flow cytometer-based functional assay for the diagnosis of HIT and explored sources of variability. Among 15 patients with confirmed HIT, HIT confirm was positive in 9 patients, resulting in a 60% sensitivity. Of 88 HIT-negative patients, HIT confirm was negative in 83 patients, leading to 94% specificity. Test modifications did not result in improved performance.



**Fig. 2.** Diagnostic accuracy of a functional, flow cytometer-based assay for the diagnosis of heparin-induced thrombocytopenia (HIT Confirm). (A) Distribution of test results (HEPLA%, percentage of CD62P positive events) according to the presence of HIT. Cut-offs are shown as dashed lines. (B) Classification table of HIT confirm according to the presence of HIT. The sensitivity was 60.0% (95% confidence interval 32.3, 83.7) and specificity was 94.3% (95% CI 87.2, 98.1). HIT was defined as a positive heparin-induced platelet activation test (HIPA).

Even though some differences exist, our results are essentially in line with previous publications. Althaus and colleagues studied 390 sera and determined the diagnostic accuracy regarding the HIPA test [16]. Sensitivity was 70%, and specificity was 75%. The most probable explanation for the higher sensitivity is that a different analyzer was used in Althaus's study; all other analytical details were similar. These observations question the reproducibility of measurements among laboratories. Vayne and colleagues conducted an artificial experiment using 5B9 monoclonal IgG antibodies mimicking human HIT antibodies and utilizing serum from 20 healthy volunteers and found a sensitivity of 83% [10]. Tardy and colleagues analyzed 228 patients and determined the diagnostic accuracy with regard to expert opinion, and found a sensitivity of 83% [18].

The strength of our study is that HIT confirm was conducted in (1) consecutive patients referred for suspected HIT ensuring a representative study cohort, (2) the patients are well characterized, (3) an established and objective reference standard was used in all patients, and (4) several test modifications were applied in order to explore sources of variability. As a limitation, the number of patients was limited, resulting in a restricted precision of the estimates. However, we do not believe that this might have influenced the results because they are similar to previous studies [10,16,18].

Several studies in different settings and using various study designs have assessed the diagnostic accuracy of the flow cytometer-based Emo-Test HIT Confirm for the diagnosis of HIT. Consistently, the sensitivity was limited (70%–83%). Considering that every fourth patient with HIT would be missed, this test is not feasible for clinical practice implementation. Of note, the sensitivity of high-quality immunoassays, some of which are available in a 24/7 service, is well above 95% [7]. Besides, we tested the effects of various test modifications, as suggested by others [16]. However, neither the use of a second, independent donor, nor washing of platelets, or filtration did improve the results. In contrast, our study suggests a high specificity (which was not observed in the study of Althaus et al.). Thus, HIT confirm can be applied to verify HIT for scientific purposes.

## 5. Conclusions

In the present cross-sectional diagnostic accuracy study, including 103 consecutive patients referred with suspected HIT, the sensitivity of the rapid cytometer-based assay HIT confirm was inferior compared to the current reference standard. This finding confirms previous studies suggesting that HIT confirm is not able to rule-out HIT in clinical practice. Several test modifications did not improve test performance. Other rapid functional assays are needed to improve diagnostic procedures in clinical practice.

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## Declaration of competing interest

All the authors state that they have no conflict of interest.

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